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Determination of Na^+/Cl^-, Na^+/HCO_3^- and $Na^+/K^+/2Cl^-$ co-transporter activity in corneal endothelial cell plasma membrane vesicles

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Abstract

Corneal endothelial cell derived plasma membrane vesicles were used to investigate the presence of Na^+/Cl^-, Na^+/HCO_3^- and $Na^+/K^+/2Cl^-$ co-transporter activity in the plasma membranes of these cells. Na^+/H^+ exchange was blocked by the presence of 1 mM amiloride in all determinations. The rate of accumulation of Na^+ in the presence of chloride or bicarbonate was not significantly different from its accumulation in the presence of acetate, thiocyanate or gluconate. The addition of K^+ to Na^+ plus Cl^- did not stimulate Na^+ accumulation into the vesicles. The present work provides no evidence for $Na^+/K^+/2Cl^-$, Na^+/Cl^- or Na^+/HCO_3^- co-transport in corneal endothelial cell plasma membrane vesicles. © 1997 Elsevier Science B.V.

Keywords: Corneal endothelium; Bicarbonate transport; Sodium transport; Membrane vesicle; Cotransporter

1. Introduction

Bicarbonate is actively transported by the corneal endothelium from corneal stroma to aqueous humour and in doing so regulates corneal hydration and consequently corneal transparency [1,2]. To move HCO_3^- across the endothelial cell requires metabolic energy [2,3], which is thought to be harnessed by the basolateral plasma membrane-bound Na^+/K^+-ATPase [2,4–6] because inhibition of Na^+/K^+-ATPase by ouabain causes complete inhibition of the endothelial transport processes [7,8]. The absence of a net Na^+ flux in the short-circuited preparation [9] and the baso-lateral location of the Na^+/K^+-ATPase [6], has led to the suggestion that Na^+ entry into the cell across the basolateral membrane and down its electrochemical gradient is by a mechanism which is coupled to and drives the transendothelial HCO_3^- flux [10]. The various models that describe net HCO_3^- transport suggest a number of possible mechanisms for coupling trans-membrane Na^+ flux and transendothelial net HCO_3^- flux. These include a direct coupling via a Na^+/HCO_3^- cotransporter [11,12], or an indirect coupling utilising a $Na^+/K^+/2Cl^-$ cotransporter further coupled to a Cl^-/HCO_3^- exchanger [10]. Other models suggest that an amiloride inhibitable Na^+/H^+ exchanger located on the baso-lateral membrane can contribute to HCO_3^- movement [13,14], although this activity has been suggested not to contribute directly to the endothelial osmotic pump [15]. To distinguish between

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the several coupling mechanisms it is necessary to investigate the transporters present in the plasma membranes of these cells.

One method that can be used to investigate transporter activity in membranes is to use isolated plasma membrane vesicles. Plasma membrane vesicles can be made essentially free of cell organelles and can provide a pure membrane sample with its associated transporters. A further advantage is that the internal and external compartments can be controlled. Their use has been successful in determining transport activity in a number of tissues other than corneal endothelium [16–20]. In this study we used corneal endothelial cell plasma membrane vesicles to investigate the mechanisms of coupling between Na^+ and HCO_3^-. By controlling the external and internal compartments of the vesicle we were able to monitor ion movement, in particular Na^+, across the membranes. We studied the uptake process at 25°C at which temperature the corneal endothelium is still able, by its transport capability, to maintain the transparency of the cornea [21]. We could find no evidence for $Na^+/K^+/2Cl^-$, Na^+/Cl^-, or Na^+/HCO_3^- co-transporters in isolated corneal endothelial cell plasma membrane vesicles.

2. Materials and methods

2.1. Preparation of plasma membrane vesicles

Bovine eyes were obtained from a local abattoir 2–4 h post-mortem. The eyes were used immediately or refrigerated at 4°C for use within 24 h.

To isolate corneal endothelial cells a small incision was made in the sclera, approximately 3 mm from the limbus. This incision was extended around the globe and the cornea with the scleral rim removed from the rest of the eyeball. The cornea was washed in 20 ml of 250 mM sorbitol to remove traces of iris pigment and unwanted tissue. One experimental batch comprised 16 ox eyes. The endothelial cells were removed by scraping and were transferred into 2 ml of 250 mM sorbitol. The cell suspension was carefully transferred to a 1 ml Potter-Elvehjem homogenizer and homogenised with four strokes of the plunger. This suspension was centrifuged using self-forming Percoll gradients under isopycnic centrifugation for 30 min at 4°C at 29,000 rpm ($80{,}000 \times g_{max}$; $60{,}000 \times g_{avg}$) [5]. Following centrifugation three distinct bands were visible in the Percoll gradient, previously identified as: band 1, the nuclear enriched fraction; band 2, the plasma membrane enriched fraction and band 3, the mitochondrial enriched fraction [5]. The plasma membrane fraction was removed and transferred into a clean centrifuge tube. The plasma membrane fraction was then centrifuged at 35,000 rpm ($118{,}000 \times g_{max}$; $90{,}000 \times g_{avg}$) for 1 h to sediment out the membranes from the Percoll. After removal of Percoll, 300 μl of the final pellet containing the membranes was resuspended in 300 μl of a buffered sorbitol solution A with a final composition of (in mM); 250, sorbitol; 0.2, $CaSO_4$; 10, $MgSO_4$; 1, amiloride; 10, Hepes; 10, Trizma base; at pH 7.5. This solution A provided the intravesicular compartment at the start of every experiment carried out in this series. Plasma membrane vesicles were formed by passing the membrane suspension four times through a 19G hypodermic needle.

2.2. Na^+ uptake into the vesicles

$^{22}Na^+$ uptake into the vesicle was measured using a rapid filtration technique [20]. To initiate the transport reaction, 10 μl of vesicle suspension (made to a concentration of 0.4 mg protein ml^{-1}) was shaken together with 10 μl of bathing solution (containing radiolabelled substrate). Final external solution concentrations depended upon the particular membrane proteins being investigated but were based upon an iso-osmotic substitution of the intra-vesicular medium (solution A above) in which salts replaced a fraction of the sorbitol. The vesicle suspension and the bathing solution were mixed in a waterbath at 25°C. The bathing solution always included at least 6 kBq of ^{22}Na in order to monitor Na uptake rates into the vesicle. The reaction was terminated after 15 s by the addition of 3 ml of ice-cold 'stop' solution A (above). The diluted ice-cold suspension was quickly filtered through a pre-wetted membrane filter (HAWP 02500, 0.45 m pore size) attached to a Millipore vacuum pump assembly under light suction. The filters were then washed with 3 ml of stop solution, removed and placed in 10 ml of scintillation fluid (Ecoscint). Each determination was performed five times and the activities were measured in a liquid scintillation counter

and were used to calculate the rate of Na^+ uptake during the 15 s exposure period. All data were corrected for non-specific trapping of ^{22}Na by the membranes and filters. To do this, uptake at 'zero time', which is that measured when the vesicle and stop solutions were added simultaneously, was subtracted from the uptake after 15 s. Vesicle protein was measured using Coomassie blue protein assay reagent, with bovine serum albumin as standard.

In control experiments it was established that $^{22}Na^+$ uptake was, within the limits of experimental error, linear for at least 15 s for the entire range of experimental conditions considered in this paper (Lane, Wigham and Hodson, unpublished data). Vesicle integrity for each experimental procedure was checked by measurement of radiolabelled L-alanine uptake into corneal endothelial cell plasma membrane vesicles, in the presence of Na^+ or K^+ containing solutions [22].

2.3. Reagents and materials

Percoll was obtained from Pharmacia LKB, Uppsala, Sweden; membrane filters from Millipore, UK; $^{22}NaCl$ from Amersham, UK; ^{22}Na acetate from NEN Dupont, USA; Ecoscint from National Diagnostics, USA. All other reagents were obtained from Sigma, Poole, UK.

3. Results

3.1. Viability

The ionic dependence of L-alanine accumulation was used to check the viability of the corneal endothelial cell plasma membrane vesicles before and after each experimental procedure. The ratio of uptake in Na^+ compared to K^+ solution after 15 s incubation was 2.4 ± 0.2 (mean $\pm$ S.D.).

3.2. Na^+ uptake in the presence of an inward Cl^- gradient

To determine the presence of Na^+/Cl^- cotransporter activity, $^{22}Na^+$ uptake was measured in the presence of an inward NaCl gradient which as a control was substituted by an inward Na acetate gradient. A number of solution concentrations were tested but the chief results are reported for 1 mM NaCl and Na acetate and for 125 mM NaCl and Na acetate which are shown in Fig. 1. In no case ($n = 5$) was there any significant difference in the uptake: there was no apparent Na^+/Cl^- co-transport activity in corneal endothelium plasma membranes under the conditions tested here. It was of interest to note that the basal Na^+ uptake exhibited some form of saturation because when the $[Na^+]$ in the bathing medium increased by $\times 125$, the uptake increased only by about $\times 26$ (from 2.3 ± 0.1 nmol Na^+/mg protein/15 s up to 62 ± 6 nmol Na^+/mg protein/15 s).

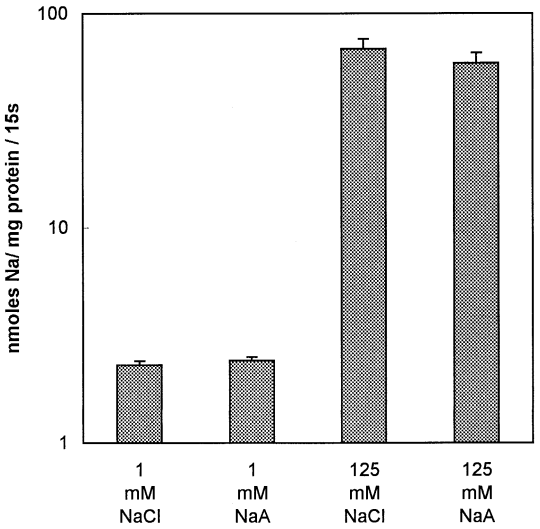

Fig. 1. The linear uptake rates of Na^+ into corneal endothelial plasma membrane vesicles under gradients of 1 mM NaCl, 1 mM Na acetate, 125 mM NaCl and 125 mM Na acetate during 15 s incubations. The ordinate is logarithmic.

3.3. Na^+ uptake in the presence of an inward HCO_3^- gradient

To look for the presence of Na^+/HCO_3^- cotransporter activity, 25 mM $KHCO_3$ was added to the external solution in order to produce an inwardly directed HCO_3^- gradient. NaCl was added to the external solution in amounts of 0.2, 1 or 40 mM (0.2 mM was used to check previously published data [23]; 1 mM was used because in preliminary experiments it was found to give the best signal to noise ratio; 40 mM was used because that it is reported as

the lowest physiological concentration of sodium which will support the endothelial pump [1]). HCO_3^- independent $^{22}Na^+$ uptake was determined by substituting $KHCO_3$ with 25 mM K^+ thiocyanate or 25 mM K^+ gluconate in the external solutions. $^{22}Na^+$ had a final activity of 0.0185 MBq in each 10 μl of bathing solution. All external solutions had appropriate reductions in their sorbitol in order to maintain isotonicity. The combined data of all 9 determinations, which were each averaged from five determinations on at least four separate samples is shown in Fig. 2. There was no significant difference between the rate of sodium accumulation in the vesicles in the presence or absence of bicarbonate over the conditions we tested.

3.4. Na^+ transport in the presence of inward K^+ and Cl^- gradients

To check for the presence of $Na^+/K^+/2Cl^-$ co-transporter activity in the membranes of the vesicles, 1 or 40 mM NaCl was added to the bathing solutions together with 0, 1 or 40 mM KCl with appropriate reductions in the sorbitol concentrations in order to maintain iso-osmolarity. If the $Na^+/K^+/2Cl^-$ co-transporter is present in the corneal endothelial cell membrane vesicles then the presence of K^+ should lead to a significant increase in uptake of $^{22}Na^+$. The rate of accumulation of $^{22}Na^+$ in 40 mM Na was 23.8 ± 2.0 nmol Na^+ mg^{-1} protein 15 s^{-1} in the absence of K^+, 25.7 ± 2.8 nmol Na^+ mg^{-1} protein 15 s^{-1} in the presence of 1 mM K^+ and 24.6 ± 2.6 nmol Na^+ mg^{-1} protein 15 s^{-1} in the presence of 40 mM K^+. None of these results were significantly different. Similarities were also found for 1 mM NaCl (Fig. 3) indicating that we found no evidence for the presence of $Na^+/K^+/2Cl^-$ activity in corneal endothelial plasma membrane under the conditions tested here.

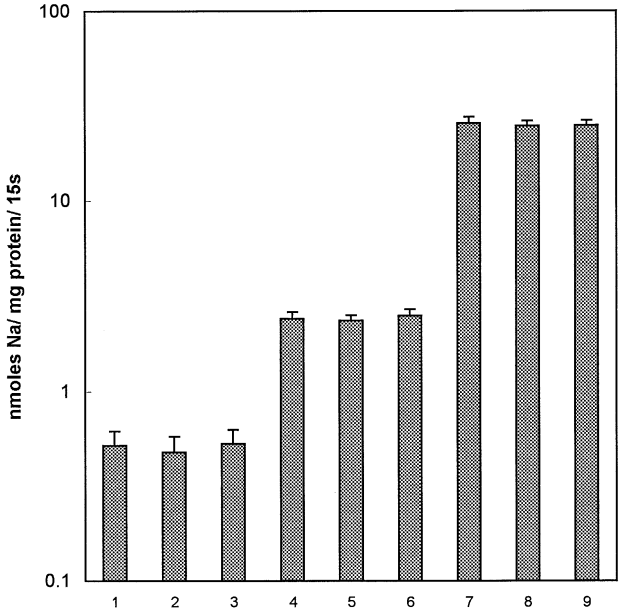

Fig. 2. The linear uptake rates of Na^+ into corneal endothelial plasma membrane vesicles under gradients of (left three) 0.2 mM Na^+ plus 25 mM bicarbonate, gluconate and thiocyanate, respectively; (middle three) 1 mM Na^+ plus 25 mM bicarbonate, gluconate and thiocyanate, respectively and (right three) 40 mM Na^+ plus 25 mM bicarbonate, gluconate and thiocyanate, respectively. The ordinate is logarithmic.

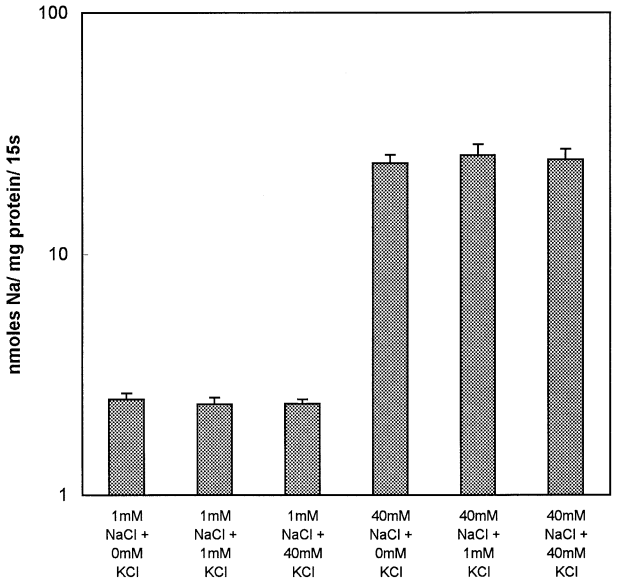

Fig. 3. The linear uptake rates of Na^+ into corneal endothelial plasma membrane vesicles under gradients of (left three) 1 mM NaCl plus 0, 1 and 40 mM KCl, respectively and (right three) 40 mM NaCl plus 0, 1 and 40 mM KCl, respectively. The ordinate is logarithmic.

4. Discussion

The integrity of the corneal endothelial plasma membrane vesicles which we prepared was investigated by looking for Na^+ dependent L-alanine up-

take. The significantly higher uptake of L-alanine in the presence of a Na^+ compared to K^+ inward gradient indicated that a constant proportion of the vesicles possess their normal orientation and that irregular osmotic disruption of the vesicles did not occur. The consistency of the uptake ratio (2.4) in all the experimental procedures we investigated indicated that comparison between addition and deletion of various salts to the bathing medium seemed not to affect the transport capabilities of the vesicles and was probably justified. The addition of amiloride has been shown previously to reduce the uptake of $^{22}N^{22}Na^+$ into corneal endothelial membrane vesicles via NHE [23] and as we were searching for sodium coupling mechanisms additional to NHE, this transport mechanism was routinely blocked in this series of experiments.

The $Na^+/K^+/2Cl^-$ cotransporter has been shown to be present in many cell types studied, including epithelial [16,24] and nonepithelial cells [25,26]. The criteria used to describe its presence include inhibition by loop diuretics, such as furosemide and bumetanide, and, more rigorously, by a demonstration that the transport of any of the component ions requires the presence of the others. Diecke et al [27] showed that in Na^+ free and Cl^- free solutions, bumetanide sensitive K^+ uptake was abolished in cultured bovine corneal endothelial cells. Also, Hodson et al [10] reported the possibility of a $Na^+/K^+/2Cl^-$ cotransporter coupled to a Cl^-/HCO_3^- exchanger to generate entry of bicarbonate into corneal endothelium. This conclusion was drawn from experiments where the Na^+ accumulation rate into corneal endothelial plasma membrane vesicles was reduced in the presence of the co-transporter inhibitor furosemide [10,23]. However, Fig. 3 shows that $^{22}Na^+$ uptake into corneal endothelial cell membrane vesicles was independent of a variety of K^+ and Cl^- gradients. The $Na^+/K^+/2Cl^-$ co-transporter appears not to be working in the corneal plasma membrane vesicles used here which suggests, in spite of furosemide sensitivity of the transport characteristics, that it may not present in corneal endothelial cells. This is consistent with other studies which showed evidence against the existence of $Na^+/K^+/2Cl^-$ transport in corneal endothelium [28,29]. Furosemide is reported to be the least specific $Na^+/K^+/2Cl^-$ cotransporter inhibitor of the loop diuretics [30]. Perhaps the furosemide effect seen in the studies reported by Hodson et al [10] were due to inhibition of Na^+ entry into the vesicles via routes other than the $Na^+/K^+/2Cl^-$ cotransporter.

The results shown in Fig. 1, indicate that there is little or no Na^+/Cl^- cotransporter activity in the corneal endothelial membrane vesicles. Although this laboratory has already reported the absence of Na^+/HCO_3^- cotransporter activity in the corneal endothelial membrane vesicles [23] it was considered to be useful to repeat these experiments using exactly the same experimental protocol as in this series. As reported previously $^{22}Na^+$ uptake into the corneal endothelial membrane vesicles was found to be independent of the nature of the anion (Fig. 3), which is inconsistent with the proposed model of Na^+/HCO_3^- cotransport in corneal endothelial cells suggested by other workers [11,12,28,31].

The present work provides no evidence for $Na^+/K^+/2Cl^-$, Na^+/Cl^- or Na^+/HCO_3^- cotransport in corneal endothelial cell plasma membrane vesicles. The nature of the coupling mechanism between HCO_3^- and Na^+, which must exist in corneal endothelial cells is still unresolved.

Acknowledgements

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